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On 9-20-02

TOWNSEND and TOWNSEND and CREW LLP

By: Linda Shaffer

PATENT
Attorney Docket No.: 016303-005310US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Sean C. Semple, *et al.*

Application No.: 09/122,588

Filed: July 23, 1998

For: LIPOSOMAL COMPOSITIONS FOR THE DELIVERY OF NUCLEIC ACID CATALYSTS

Examiner: M. Schmidt

Art Unit: 1635

Declaration of Sandra K. Klimuk, Ph.D. Under 37 C.F.R. §1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Sandra K. Klimuk, Ph.D. being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I am currently a Research Scientist at Inex Pharmaceuticals Corporation (Burnaby, Canada), a biotechnology company whose primary focus is the development of cancer treatments that are based on its propriety drug delivery platform and that are more effective and have fewer side effects than conventional cancer treatments. I have been a Research Scientist since

January 2001. Prior to this position, I was a NSERC Industrial Research Fellow at Inex Pharmaceuticals Corporation from September 1999 through January 2001.

3. In May of 1991, I graduated from the University of British Columbia (Vancouver, Canada) with a Bachelor of Science degree in Animal Biology. In July of 1999, I was awarded my Ph.D. in Biochemistry and Molecular Biology from the University of British Columbia (Vancouver, Canada). My graduate research focused on the development of several different methodologies for encapsulating antisense oligonucleotides and ribozymes into liposomes. In the course of my graduate studies, I developed several acute and chronic murine inflammation models to examine the pharmacokinetics, toxicity and efficacy of various liposomal DNA formulations *in vivo*. The title of my thesis was "*Liposomal Encapsulation Enhances the Anti-Inflammatory Activity of an ICAM-1 Antisence Oligonucleotide.*"

4. Attached hereto as Exhibit A is a true copy of my *curriculum vitae* and a list of publications of which I am an author or co-author.

5. I am a named inventor on the above-referenced patent application. I have read and am familiar with the contents of this patent application. In addition, I have read the Office Action, mailed May 29, 1999, and the Final Office Action, mailed June 5, 2000, received in the above-referenced patent application. It is my understanding that the Examiner has raised a concern about whether the data that demonstrates the ability of the claimed liposomal ribozyme compositions to effectively treat neoplasia in mice can be readily correlated with utility in humans and mammals other than mice. For the reasons set forth herein, the Examiner's concern is overcome.

6. The present invention is directed, in part, to methods for treating neoplasia in a mammal using pharmaceutical compositions comprising a PEG-ceramide conjugate, a lipid, and a nucleic acid catalyst (*i.e.*, a ribozyme). Such formulations have been found to be unusually effective in delivering nucleic acid catalysts to neoplastic cells *in vivo*, thereby providing therapeutic results.

7. It is my opinion that the mouse studies set forth in the specification (*see,* Exhibit B, Examples 3, 4, 5, and 6) are accurate predictors of the ability of the presently claimed

liposomal ribozyme compositions to function effectively in human and mammals other than mice. There is a large body of scientific literature describing the use of mouse disease models to elucidate the pathogenesis of various diseases and to identify drug targets. In view of this, it is clear that other scientists agree that work in experimental mammals is of critical importance and relevance to other mammals, including humans.

8. Even those references that describe the limitations of mouse models still clearly accept the importance of research in mice. For example, the article cited by the Examiner regarding this point in the first Office Action, Crystal (1995), simply points out that, occasionally, predictions based on work in mice do not prove true in humans. As stated by Crystal:

> *Humans are not simply large mice.* There have been several ***surprise examples***, in which predictions from gene transfer studies in experimental animals have not been borne out in human safety and efficacy trials. (Exhibit C, page 40; non-bold italics in original, bold italics added).

By highlighting the existence of occasional "surprise examples" in which these predictions are not borne out, Crystal simply notes that the predictive value of such studies is not absolute. However, in doing so, Crystal in no way challenges the universal position among scientists, such as myself, that work performed in experimental mammals is relevant to other mammals and is, most often, an accurate predictor of therapeutic efficacy in other mammals, such as humans.

9. Moreover, in addition to the demonstration of efficacy in a Lewis lung carcinoma mouse model presented in the specification (*see,* Exhibit B, Example 6), it has recently been demonstrated that the anti-VEGFR ribozyme also effectively inhibits tumor growth and metastasis in a human colorectal carcinoma mouse model (*see,* Exhibit C). In my opinion, this demonstration of efficacy in mouse models for two distinct types of cancer strongly supports the conclusion that the liposomal ribozyme compositions of the present invention will also function effectively in other mammals, including humans. As discussed above, the close correlation between efficacy in any particular experimental mammal and efficacy in other mammals is widely accepted by scientists. In view of this, it is clear that demonstration of efficacy in two mouse models, each closely modeling different aspects of the pathology of other mammals, provides even further support for the utility of such compositions in other mammals, such as humans.

10. It should be noted that the studies described in Exhibit C were conducted with an unencapsulated form of the ribozyme, rather than the liposomal composition of this invention. However, since liposomal encapsulation has been found to enhance the therapeutic effect of a ribozyme by protecting it from degradation, it can be concluded that a liposomal composition of the ribozyme would be just as, if not more, effective than the unencapsulated form. As such, as described above, these studies support, in my opinion, the idea that efficacy of these liposomal ribozyme compositions in mice correlates with efficacy in other mammals, such as humans.

11. In conclusion, it is my opinion that studies evaluating therapeutic efficacy in experimental mammals, such as mice, are accurate predictors of therapeutic efficacy in other mammals, including humans. The Examiner has provided no evidence to contradict this position universally held among scientists. Moreover, it is my opinion that the mouse model data provided in the specification, either alone or in combination with the additional data provided in Exhibit C, strongly indicates that the liposomal ribozyme compositions of the present invention would also function effectively in other mammals, including humans. We have unequivocally established that the liposomal ribozyme compositions of this invention inhibit tumor growth in mouse models *in vivo*. Based on the usefulness of such mouse models, I have no reason to believe that the claimed compositions would function differently in other mammals, including humans. As such, it is my opinion that the *in vivo* mouse data set forth in the specification is an accurate predictor of the ability of the claimed liposomal ribozyme compositions to treat neoplasia *in vivo* in humans.

The declarant has nothing further to say.

Sandra K. Klimuk, Ph.D.

Sept 10/02
Date

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EDUCATION

Sept. 1992 – July 1999 **Ph.D.** (Biochemistry and Molecular Biology), University of British Columbia, Vancouver, Canada. **Thesis title: *Liposome encapsulation enhances the anti-inflammatory activity of an ICAM-1 antisense oligonucleotide.***

Sept. 1987 - May 1991 **B.Sc.** (Animal Biology), University of British Columbia, Vancouver, Canada.

RESEARCH EXPERIENCE

Jan. 2001 – Present **Research Scientist**, Inex Pharmaceuticals Corporation, Burnaby, Canada.

Sept. 1999 – Jan. 2001 **NSERC Industrial Research Fellow**, Inex Pharmaceuticals Corporation, Burnaby, Canada.

Sept. 1992 – Aug. 1999 **Graduate Student**, Supervisor: Dr. M. J. Hope, Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, Canada.

- Developed several methodologies for encapsulating antisense oligonucleotides and ribozymes into liposomes. (Collaborations with Inex Pharmaceuticals, Isis Pharmaceuticals and Ribozyme Pharmceuticals Inc.)
- Established acute and chronic murine inflammation models to examine the pharmacokinetics, toxicity, and efficacy of various liposomal DNA formulations *in vivo*.

May 1992 - Sept. 1992 **MRC Summer Studentship**, Supervisor: Dr. M. J. Hope, Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, Canada.

- Established a rabbit model of hyperlipidemia to examine the pharmacokinetics and efficacy of liposomal therapy to regress atheroma.

Sept. 1991 - May 1992 **Directed Research Studies**, Supervisor: Dr. M. J. Hope, Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, Canada.

- Kinetic analysis of the transbilayer movement and exchange of cholesterol and cholesterol sulphate from the outer monolayer of liposomes.

Sept. 1990 - May 1991 **Directed Research Studies**, Laboratory of Dr. A. M. Perks, Department of Zoology, University of British Columbia, Vancouver, Canada.

EXHIBIT A

- Examined the effect of metabolic inhibitors on fetal guinea pig lung liquid production.

AWARDS

Natural Sciences and Engineering Research Council of Canada (NSERC) - Industrial Research Fellowship (IRF)	2000-Present
British Columbia Science Council Graduate Research Engineering and Technology (GREAT) Award	1994-1997
University of British Columbia Travel Award,	1996
Medical Research Council of Canada Summer Studentship,	1992

PROFESSIONAL MEMBERSHIPS

American Association for Cancer Research, Active Member

American Association of Immunologists, Active Member

PUBLICATIONS (Published or in Press)

1. Semple, S.C., **Klimuk, S.K.,** Harasym, T.O., Dos Santos, N., Ansell, S.M., Wong, K.F., Maurer, N., Stark, H., Cullis, P.R., Hope, M.J. and Scherrer, P. Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel small multilamellar vesicle structures. *Biochim. Biophys. Acta.*, 1510(1-2):152-166 (2001).
2. **Klimuk, S.K.**, Semple, S.C., Nahirney, P.N., Mullen, M.C., Bennett, C.F., Scherrer, P. and Hope, M.J. Enhanced anti-inflammatory activity of a liposomal intercellular adhesion molecule-1 antisense oligonucleotide in an acute model of contact hypersensitivity. *J. Pharmcol. Exp. Therap.* 292(2):480-488 (2000).
3. Semple, S.C., **Klimuk, S.K.**, Harasym, T.O. and Hope, M.J. Lipid-based formulations of antisense oligonucleotides for systemic delivery applications. *Methods Enzymol.* 313:322-341 (2000).
4. **Klimuk, S.K.**, Semple, S.C., Scherrer, P. and Hope, M.J. Contact hypersensitivity: a simple model for the characterization of disease-site targeting by liposomes. *Biochem. Biophys. Acta* 1417(2):191-201 (1999).
5. Webb, M.S., **Klimuk, S.K.**, Semple, S.C. and Hope, M.J. Lipid-based carriers for the systemic delivery of antisense drugs. In: *Manual of Antisense Methodology.* Hartmann, G. and Endres, S., Eds. Kluwer Academic Publishers. Pages 167-190 (1999).
6. Ansell, S.M., Kojic, L.D., Hankins, J.S., Sekirov, L., Boey, A., Lee, D.K., Bennett, A.R., **Klimuk, S.K.**, Harasym, T.O., Dos Santos, N., and Semple, S.C. Application of oligo-(14-amino-3,6,9,12-tetraoxatetradecanoic acid) lipid conjugates as steric barrier molecules in liposomal formulations. *Bioconjugate Chem.* 10(4):653-666 (1999).
7. Rodriqueza, W.V., **Klimuk, S.K.,** Pritchard, P.H. and Hope, M.J. Cholesterol mobilization and regression of atheroma in cholesterol-fed rabbits induced by large unilamellar vesicles. *Biochem. Biophys. Acta* 1368(2):306-320 (1998).
8. Rodriqueza, W.V., Wheeler, J.J., **Klimuk, S.K.**, Kitson, C.N. and Hope, M.J. Transbilayer movement and net flux of cholesterol and cholesterol sulfate between liposomal membranes. *Biochemistry* 34(18):6208-6217 (1995).

MANUSCRIPTS (Submitted or in Preparation)

9. Sandberg, J.A., Min, J.J., Jensen, K.L., Bouhana, K.S., Gallegos, A.M., **Klimuk, S.K.**, Semple, S.C., Scherrer, P., Hope, M.J., Parry, T.J. and Reynolds, M.A. Lipid-based carriers enhance the biodistribution and efficacy of anti-angiogenic ribozyme in a murine Lewis lung carcinoma model. *Clin. Cancer Res.*, submitted.
10. Semple, S.C., Harasym, T.O., Clow, K., Kojic, L., Ansell, S.M., **Klimuk, S.K.** and Hope, M.J. Immunogenicity and rapid plasma elimination of non-viral delivery systems for gene and antisense therapy. *Proc. Natl. Acad. Sci. USA*, submitted.
11. Semple, S.C., Ahkong, L., Leng, E., Mui, B., Hope, M.J. and **Klimuk, S.K.** Pre-clinical anti-tumor activity of liposomal topotecan: increased efficacy and therapeutic index of liposomal topotecan in murine and human xenograft tumor models compared to free drug. *Clin. Cancer Res.*, in preparation.
12. Semple, S.C., Bramson, J.L., Ludkovski, O., Clow, K., **Klimuk, S.K.**, Hope, M.J. and Harasym, T.O. Enhanced potency of phosphorothioate c-myc antisense oligodeoxynucleotides in murine and human tumor models upon systemic administration of stabilized antisense-lipid particles. *Antisense Nucl. Acid Drug Des.*, in preparation.

PUBLISHED ABSTRACTS

1. **Klimuk, S.K.**, Yuan, Z.-N., Ansell, S.M., Semple, S.C. Characterization of a liposomal cancer vaccine delivery system containing immunostimulatory oligonucleotides. *Proc. Amer. Assoc. Cancer Res.* 43, 447, 2002
2. Yuan, Z.-N., **Klimuk, S.K.** and Semple, S.C. Mucosal immune responses induced by immunostimulatory oligonucleotides are enhanced when formulated in lipid particles. *FASEB J.* 16(4), A680, 2002.
3. Semple, S.C., **Klimuk, S.K.,** MacLachlan, I., Leng, E., Mui, B. and Hope, M.J. Pre-clinical evaluation of liposomal topotecan: increased efficacy and therapeutic index in murine and human xenograft tumor models compared to free drug (#2015). *Proc. Amer. Assoc. Cancer Res.* 42:374, 2001
4. Semple, S.C., **Klimuk, S.K.,** Mui, B. and Hope, M.J. Encapsulation of immunostimulatory oligonucleotides in lipid particles dramatically increases immune responses and leads to effective tumor immunotherapy in mice (#4405). *Proc. Amer. Assoc. Cancer Res.* 42:820, 2001
5. Semple, S.C., Bramson, J.L, Ludkovski, O., Clow, K., Dos Santos, N., Joshi, P. **Klimuk, S.K.**, Hope, M.J. and Harasym, T.O. Pre-clinical studies with stabilized antisense-lipid particles (SALP) containing c-myc antisense oligonucleotides. *J. Liposome Res.* 10(2/3), 277-278, 2000.
6. Semple, S.C., Clow, K., Harasym, T.O., Airriess, R.A., **Klimuk, S.K.** and Hope, M.J. Immunogenicity and rapid blood elimination of PEG-liposomes containing entrapped nucleic acid upon repeat administration. *J. Liposome Res.* 10(2/3), 278, 2000.
7. Ahkong, L., Airriess, R., Harasym, T., Hope, M., **Klimuk, S.**, Leng, E., MacLachlan, I., Semple, S.C., Tam, P. and Hope, M.J. Pre-clinical studies with liposomal mitoxantrone: formulation, pharmacokinetics, toxicity and efficacy. *J. Liposome Res.* 10(2/3), 199-200, 2000.
8. Semple, S.C., **Klimuk, S.K.,** Harasym, T.O., Scherrer, P., Dos Santos, N., Ansell, S.M., Lutwyche, P. and Hope, M.J. Stabilized antisense-lipid particles (SALP) for systemic applications: generation, characterization and in vivo properties. *J. Liposome Res.* 8(1), 104-105, 1998.

POSTER PRESENTATIONS

1. **Klimuk, S.K.**, Semple, S.C., Scherrer, P., Bennett, C.F. and Hope, M.J. *Enhanced delivery and in vivo efficacy of lipid-encapsulated ICAM-1 antisense oligodeoxynucleotide in a murine model of ear inflammation.* IBC's Seventh Annual International Conference on Cell Adhesion Molecules and Matrix Proteins: Advances in Therapeutic Application and Development, San Franciso, CA, USA, May, 1997.

2. **Klimuk, S.K.**, Semple, S.C., Scherrer, P., Bennett, C.F. and Hope, M.J. *Enhanced anti-inflammatory activity of liposomal ICAM-1 antisense oligonucleotides.* Michael Smith Symposium, Vancouver, BC, April 1997.

SELECTED ABSTRACTS

1. Semple, S.C., Bramson, J.L., Raney, S.G., **Klimuk, S.K.,** Hope, M.J. and Mui, B. *Stabilized antisense-lipid particles (SALP) strongly enhance the immune stimulation of CpG oligonucleotides: an "artifical virus" approach to cancer vaccines.* Cancer Vaccines 2000 – Cancer Research Institute, New York, NY, USA, October 2000.
2. **Klimuk, S.K.**, Semple, S.C., Scherrer, P., Bennett, C.F. and Hope, M.J. *Inhibition of the inflammation response by lipid-encapsulated ICAM-1 antisense oligonucleotide.* 2nd Canadian Symposium on Gene Therapy, Vancouver, B.C., Canada, 1997.
3. **Klimuk, S.K.**, Semple, S.C., Scherrer, P., Bennett, C.F. and Hope, M.J. *Enhanced anti-inflammatory activity of liposomal ICAM-1 antisense oligonucleotides.* International Congress: Therapeutic Oligonucleotides, Rome, Italy, June, 1996.
4. **Klimuk, S.K.**, Semple, S.C., Scherrer, P. and Hope, M.J. *Increased circulation lifetime and accumulation of liposomal antisense at a site of inflammation.* International Congress: Therapeutic Oligonucleotides, Rome, Italy, June, 1996.
5. Semple, S.C., **Klimuk, S.K.**, Scherrer, P. and Hope, M.J. *Liposome encapsulation of antisense oligonucleotides decreases complement activation.* International Congress: Therapeutic Oligonucleotides, Rome, Italy, June, 1996.

PATENTS

1. Enhanced efficacy of liposomal antisense delivery. Inventors: **Klimuk, S.K.**, Semple, S.C., Scherrer, P. and Hope, M.J. Owner: University of British Columbia. WO9746671.
2. High efficiency encapsulation of charged therapeutic agents in lipid vesicles. Inventors: Semple, S.C., **Klimuk, S.K.**, Harasym, T.O., Hope, M.J., Ansell, S.M., Cullis, P.R., Scherrer, P. and Debeyer, D. Owner: INEX Pharmaceuticals Corp. WO9851278.
3. Liposomal compositions for the delivery of nucleic acid catalysts. Inventors: **Klimuk, S.K.**, Scherrer, P., Hope, M.J., Zhang, Y.P., Reynolds, M.A., Min J. and Semple, S.C. Owner: Ribozyme Pharmaceuticals Corp. WO9904819.
4. Compositions for stimulating cytokine secretion and inducing an immune response. Inventors: Semple, S.C., Harasym, T.O., **Klimuk, S.K.,** Kojic, L.D., Bramson, J.L., Mui, B. and Hope, M.J. Owner: Inex Pharmaceuticals Corp. WO0115726.
5. Charged therapeutic agents encapsulated in lipid particles containing four lipid components. Inventors: Semple, S.C., **Klimuk, S.K.**, Harasym, T.O., Hope, M.J., Ansell, S.M., Cullis, P.R., Scherrer, P. and Debeyer, D. Owner: INEX Pharmaceuticals Corp. US6287591.

were purified from unencapsulated material using an FPLC column packed with DEAE sepharose CL-6B. Efficiency of encapsulation was determined by HPLC analysis an a C18 column (gradient of 4-18% acetonitrile in water). Lipid concentration was determined by measuring cholesterol concentration using a cholesterol quantitation assay (Sigma Chemicals) following the manufacturers instructions. In pharmacokinetic experiments the tritiated CHE (^{3}H-cholesteryl hexadecyl ether) was used to track and quantitate lipid concentration and 32P was used to track the ribozyme concentration. Radioisotopes were quantitated in a scintillation counter.

B. Example 2: Formation of Liposome Encapsulated Ribozyme by Bligh & Dyer Extraction

DOTAP (2.44 mg), EPC(2.75 mg), PEG-Ceramide-C8 (1.31 mg) were combined together suspended in chloroform in a glass test tube. The lipids were then dried down under argon gas. The lipid mixture was then suspended in a mixture of chloroform (0.73 ml) and Methanol (1.54 ml). A hammerhead ribozyme with a ^{32}P tracer (1 mg) suspended in water (0.73 ml) was then added to the lipid containing organic solvents. Vortexing the solution resulted in a monophasic solution of $CHCl_3$, MeOH and H_20 (1:2.1:1). Chloroform (0.75 ml) and water (0.75 ml) was then added to cause phase separation of the organic and aqueous components of the solution. The mix was then vortexed for 1 minute and then centrifuged at 2000 RPM for 5 minutes. The aqueous layer was then removed and then examined for ribozyme content by reading the absorbance at 260 nm wavelength using a spectrophotometer. The organic phase was dried down under argon gas and then rehydrated in normal saline. Ribozyme content was determined by counting a sample of the liposome preparation in a scintillation counter.

C. Example 3: Pharmacokinetic Analysis of a ribozyme-liposomal formulation in Neonatal Murine Eyes

Seven day old (P7) neonatal mice and their nursing dams were placed into an oxygen rich chamber (75% O_2/25% N_2) with *ad libitum* food and water. Five days later (P12), they were removed from the chamber and injected immediately (day zero group) or allowed to recover five days and injected on P17 (day five group). Liposome formulated and non-formulated ribozyme was administered via intravitreal injection on

P12 or P17. The neonatal mice, anesthetized with 40 μl 2.5% Avertin, received a single intravitreal bolus of 5 μg of VEGF-R-1 ribozyme (supplemented with 10 x 10^5 cpm/μg ^{32}P VEGF-R-1 ribozyme; Figure 2) formulated with EPC-DOTAP:PEG liposomes or non-formulated VEGF-R-1 ribozyme (supplemented with 10 x 10^5 cpm/μg ^{32}P VEGF-R-1 ribozyme) in sterile saline. Neonates treated with ^{32}P VEGF-R-1 ribozyme were euthanized with CO_2 at 0.5, 4, 24, 48, 72 hours after ribozyme administration. Upon cessation of breathing, the chest cavity was opened and blood sampled (150-250 μl) from the heart. Sampled blood was added to a heparinized microfuge tube and centrifuged for 10 minutes to separate plasma and blood cells. Retina, capsule, kidney and liver were dissected from each and immediately frozen on dry ice. Frozen tissue from ^{32}P VEGF-R-1 ribozyme treated neonates was pulverized and digested in a proteinase K containing buffer (100 mM NaCl, 10 mM tris (pH 8), 25 mM EDTA, 10% SDS). A portion of the sample was added to scintillant and counted. Undiluted plasma was added to scintillant and counted. Tissue samples having greater than one hundred cpm per 50 μl of digested sample were analyzed for the presence and the percent of intact ribozyme via PAGE and phosphorimaging analysis.

Concentrations of intact ribozyme in hyperoxic treated neonatal mouse retina and capsule are shown in Figure 3. Intact ribozyme was detected in the retinas and capsules of the neonates through 72 hours (10 ng/mg) after injection of formulated ribozyme with 75-95% of the radioactivity associated with intact ribozyme (Figure 4). Much lower concentrations of intact ribozyme were detected in the retina and capsule of the neonates administered free ribozyme (0.05-0.5 ng/mg at 72 hours. Concentrations of intact ribozyme in hyperoxic treated neonatal mouse plasma after intravitreal administration (on day zero and on day five) free or formulated ribozyme are shown in Figure 5. Intact ribozyme was detected in plasma from animals treated with free ribozyme (15 ng/ml at 24 hours. However, there was no detectable intact ribozyme in the plasma of the neonates receiving liposome formulated ribozyme (Figure 6). Tissue concentrations in the liver and kidney after intravitreal injection of formulated or free ribozyme are shown in Figure 7. Intact ribozyme was detected in the livers of the neonates 72 hours after injection of formulated ribozyme (0.05 ng/mg) or free ribozyme (0.001 ng/mg). In kidneys of the neonates in the day zero group, intact ribozyme was detected only through the 4 hour time point (0.03 ng/mg) after administration of free

ribozyme. However, intact ribozyme was detected in kidneys through 4 hours and then again at the 48 and 72 hours after administration of formulated ribozyme (Figure 8).

Area under the concentration time curve (AUC) was calculated as an indication of tissue ribozyme exposure. As shown in Table II, there was a 25 to 37 fold increase in the AUC over the 72 hour time course when the injected ribozyme was formulated with EYPC:DOTAP-PEG C8 liposomes compared with free ribozyme. There was also a 9 to 11 fold increase in ribozyme exposure of the capsule with the formulated ribozyme. AUC calculations for kidney, liver and plasma were not performed due to intermittent detection of intact ribozyme.

TABLE II. Retina and capsule areas under the curve (AUC) from hyperoxic treated meonatal mouse ribozyme tissue concentrations after intravitreal administration of 5 μg VEGF-R-1 ribozyme (supplemented with 10 x 10^6 cpm ^{32}P VEGF-R-1) formulated in an EYPC:DOTAP:PEG liposome or non-formulated (EYPC = egg yolk phosphatidylcholine). Mice were administered ribozyme either immediately upon their removal from the hyperoxic chamber or five days after their removal from the hyperoxic chamber.

		Day 0		Day 5	
Tissue	Formulation	$AUC_{0\text{-}72hr}$	PEG-C8 AUC / Free AUC	$AUC_{0\text{-}72hr}$	PEG-C8 AUC / Free AUC
Retina	Free PEG-C8	71 2600	37	65 1649	25
Capsule	Free PEG-C8	70 740	11	91 850	9
Plasma	Free PEG-C8	515 ND		413 ND	

D. Example 4. Blood Clearance Screen of Intravenously Administered Liposomal formulations

Female C57B1/6J weighing 20-25 g were used to screen various formulations of liposome encapsulated ribozyme. The following formulations were prepared using the protocol in example 1: EPC:CHOL (55:45), Shingomyelin(SM):EPC:CHOL (33:33:33), and EPC:CHOL:DODAC:PEG-ceramide-C20 (50:25:15:10). In these experiments the

ribozyme included a tracer of ^{32}P labeled ribozyme and CHE was used to track and quantitate the lipid. A single i.v. made via the tail vein. Each dose contained about 3 μmoles total lipid and between 25-50 μg of VEGF-R-1 ribozyme in a volume of 100 μL. The time points observed were 15 minutes, 2 hours, 4 hours and 24 hours. At each time point animals were euthanized with CO_2. Upon cessation of breathing, the chest cavity was opened and blood sampled (200-500 μL) from the heart. Sampled blood was added to a heparinized microfuge tube and centrifuged for 10 min to separate plasma and blood cells. Plasma samples were treated with proteinase K containing buffer. A portion of the sample was added to scintillant and counted. The sample was resolved via 15% polyacrylamide gel electrophoresis and quantitated using phosphorimager analysis.

The data (Figure 9) indicated that of the three formulations tested, the best was the formulation which contained PEG-Ceramide. The PEGylated liposomes were present in large quantities even after 24 hours suggesting that the elimination half life may be in the order of hours if not days.

E. Example 5: Pharmacokinetic Evaluation of Liposome Encapsulated Ribozymes in Lewis Lung Carcinoma Model

Female C57B1/6J weighing 20-25 g were implanted with a 0.1 mL suspension of Lewis Lung carcinoma tumor cells (5 x 10^6 cells/mL in normal saline), injected subcutaneously into the right flank. Tumors were allowed to grow for 17 days prior to dosing with liposomal ribozyme formulations. Formulations were made using the protocol described in example 1. EPC:CHOL:DODAC:PEG-ceramide-C20 (50:25:15:10), EPC:CHOL:DODAC:PEG-ceramide-C8 (50:25:15:10) and EPC:CHOL liposomes were made with CHE as a tracer. Ribozyme contained ^{32}P labeled ribozyme tracer. A single i.v. bolus injection was made via the tail vein. Injections may also be made via the jugular vein. Each "liposome formulation" dose contained about 3 μmoles total lipid and between 25-50 μg of VEGF-R-1 ribozyme in a volume of 100 μL. After dosing and at the indicated harvest times (2, 6, 24, 48, and 72 hours), animals were euthanized with CO_2. Upon cessation of breathing, the chest cavity was opened and blood sampled (200-500 μL) from the heart. Sampled blood will be added to a heparinized microfuge tube and centrifuged for 10 minutes to separate plasma and blood cells. Following blood sampling, animals were perfused with sterile saline through the heart until the liver is cleared of blood (10 mL). The tumor and the adjacent vascular

tissue were surgically removed, snap frozen in liquid nitrogen and transferred to a tared culture tube. Tissue was then pulverized or homogenized and then digested with proteinase K containing buffer. A portion of the sample was added to scintillant and counted. The sample was analyzed via PAGE and phosphorimaging. Liposomes containing PEG-Cer-C20 lipid performed better than PEG-Cer-C14 or EPC:CHOL liposomes, based on plasma levels of intact ribozyme (Figure 10). On the other hand, the data for the PEG-Cer-C20 containing liposome about 7% of the administered ribozyme dose was detected as intact ribozyme in plasma after 72 h. Tumor exposure was significantly enhanced for PEG-ceramide-C20 containing liposomal formulations compared to the other ribozyme formulations (Figure 11). The degree of enhancement correlated roughly with plasma levels (Figure 9). Quantitations of ^{32}P-ribozyme and ^{3}H-CHE lipid tracer indicated that the liposomes circulate in blood mostly intact with minimal degradation. Similar clearance profiles were also observed in primary tumor tissue (Figure 12).

Ribozyme stability in tumor tissue was measured after resolving samples by polyacrylamide gel electrophoresis (PAGE) as described above. Stability was measured as the percent of total radioactivity that still remained as full length ribozyme. Ribozymes delivered using PEG-cer-C20 liposomes were 85-90% intact through 24 hours. The ribozymes delivered using the other two formulations were approximately 30% intact after just 6 hours (Figure 14).

F. Example 6: Ribozyme-efficacy in C57 Mice

Sustained tumor growth and metastasis depend upon angiogenesis. In fact, the appearance of vessels in a growing tumor is correlated with the beginning of metastatic potential. Several studies have shown that antiangiogenic agents alone or in combination with cytotoxic agents reduce lung metastases and/or primary tumor volume in the Lewis lung and B-16 melanoma models (Bergstrom, *et al.*, 1995, *Anticancer Res.*, 15:719-728; Kato, *et al.*, 1994, *Cancer Res.*, 54:5143-5147; O'Reilly, *et al.*, 1994, *Cell*, 79:315-328; Sato, *et al.*, 1995, *Jpn. J. Cancer Res.*, 86:374-382).

A major factor implicated in the induction of solid tumor angiogenesis is vascular endothelial growth factor (VEGF; Folkman, 1995, *supra*). Several human tumors have been shown to synthesize and secrete. With regard to treating lung metastasis, VEGF and VEGF receptors of both subtypes and their expression are

upregulated in the lung under conditions of hypoxia (Tuder, *et al.*, 1994, *J. Clin. Invest.*, 95:1798-1807). This may lead to neovascularization which provides the means by which tumor cells gain access to circulation (Mariny-Baron and Marmé, 1995). Thus, VEGF and its receptors may be important targets in the treatment of metastatic disease.

It has recently been shown that a catalytically active ribozyme targeting flt-1 RNA inhibits VEGF-induced neovascularization in a dose-dependent manner in a rat cornmeal model of angiogenesis (Cushman, *et al.*, 1996, Angiogenesis Inhibitors and Other Novel Therapeutic Strategies for Ocular Diseases of Neovascularization, IBC Conference Abstract). Testing with cytotoxic agents in combination with antiangiogenic ribozymes (for example VEGF-R-1 ribozyme; Figure 1) may also prove useful.

C57/BL6 female mice were instrumented with jugular catheters three days, after receiving a subcutaneous inoculation of $5x10^5$ cells Lewis lung carcinoma cells (highly metastatic variant) in a volume of 0.1 ml saline. Catheters (PE50) were implanted in the jugular vein and exteriorized for daily bolus administration. Each dose of EPC:Cholesterol:PEG-Cer-C20:DODAC (50:25:15:10) formulated VEGF-R-1 ribozyme offered to the mice was 1 mg ribozyme/ kg body wt. The liposome formulation was prepared using the Reverse Phase Evaporation method. Liposomes were injected by a hamilton syringe into the catheter and the catheter tubing was flushed using 100 μl of saline. Animals were not treated on days 18-25 after tumor implantation. Tumors were measured with a microcaliper on days 2-25 every other day to determine tumor growth. Tumor volume was determined by the following formula: $[length*(width)^2]/2$. Twenty five days following inoculation, animals were euthanized and tumors removed and weighed. To preserve tumors for possible quantitation of ribozyme content, tumors were quickly frozen in liquid nitrogen and stored at -70°C. Lungs were removed and weighed and macrometastasis counted under 4x magnification using a Leitz dissecting microscope. The data as shown in Figure 13 indicates that liposome encapsulated ribozyme inhibited tumor growth during the duration of dosing. Following cessation of ribozyme dosing the data suggests an increase in the rate of tumor growth.